Remarks

The Amendments

Claims

Claim 9 has been amended to recite a step of "comparing the fluorescence of the monitor protein that has been reacted with the test protein to a monitor protein that has not been reacted with the test protein." The amendment is supported by the specification which discloses that "a difference in the absorbance patterns was observed for reactions in which A kinase [a test protein] was added." (Page 23, lines 22-24.)

Claim 13 has been amended to recite that the phosphorylation region of the monitor protein "undergoes a conformational change upon phosphorylation." The specification supports this amendment at page 9, lines 8-10: "[A] 'phosphorylation region' means a region comprising an amino acid residue to be phosphorylated and capable of changing its conformation by phosphorylation of the amino acid residue." Claim 13 has also been amended to recite that the "conformational change causes a change of the intensity of emitted fluorescence of the monitor protein" in place of "phosphorylation of the phosphorylation region causes a change of fluorescence of the monitor protein." This amendment is supported in the specification which discloses:

> [A]n amino acid residue in a phosphorylation region is phosphorylated to alter the conformation of the phosphorylation region, and a measurement protein pair prepared at both the ends of the phosphorylation region interacts to show a measurable property . . . For example, proteins emitting fluorescence by interacting with each other, such as BSGFP and RSGFP of *Aequorea victoria*, can preferably be used.

Page 10, lines 9-18.

Specification

The specification has been amended to disclose that figures 4, 5, and 6 show the effect of protein kinase A on "fluorescence intensity as a function of wavelength" in place of "absorption wavelength." The x and y axes of figures 4, 5, and 6 support this amendment. Pages 5 and 23 of the specification have been similarly amended.

None of these amendments to the claims and specification introduces new matter, or requires further search or consideration. The amendments to the claims were not made earlier because applicants believed that the amendments and comments filed in the response to Office Action on September 8, 2003 were sufficient to overcome the rejections. The amendments to the specification were not made earlier because the errors were first identified in the final Office Action. Applicants believe that the amendments place the application in condition for allowance.

The Objections to Specification

The specification, *e.g.*, at figures 4-6, is objected to because it improperly discloses that a test protein affects the "absorption wavelength" of a monitor protein. The specification has been amended to correctly disclose that a test protein exerts a change in a monitor protein's "fluorescence intensity as a function of wavelength."

Withdrawal of this objection to the specification is respectfully requested.

The Rejection of Claim 9 Under 35 U.S.C. § 112, Second Paragraph

Claim 9 has been rejected under 35 U.S.C. § 112, second paragraph as being indefinite for omitting an essential step.

Claim 9 is directed to a method for measuring phosphorylation ability of a test protein. The method comprises a step of reacting the test protein with a monitor protein and a step of measuring fluorescence of the monitor protein. The Office Action asserts that claim 9 omits a step of "measuring fluorescence of the monitor protein before and after reaction with a test protein." To expedite prosecution, applicants have amended claim 9 to include a step of "comparing the fluorescence of the monitor protein that has been reacted with the test protein to a monitor protein that has not been reacted with the test protein."

Withdrawal of this rejection to claim 9 is respectfully requested.

The Rejection of Claims 4, 5, 9, and 13 Under 35 U.S.C. § 112, First Paragraph

Claims 4, 5, 9, and 13 are rejected under 35 U.S.C. § 112, first paragraph as containing subject matter which was not sufficiently described in the specification. The rejection is respectfully traversed.

Claims 4, 5 and 9 depend from claim 13. Claim 13 is directed to a monitor protein for measuring protein phosphorylation. The monitor protein comprises (a) a phosphorylation region that undergoes a conformational change upon phosphorylation and (b) a pair of fluorescent proteins. Each fluorescent protein of the pair is bound to an opposite end of the phosphorylation region. The conformational change causes a change of the intensity of emitted fluorescence of the monitor protein. Claim 4 further recites that the pair of fluorescent proteins comprises a red-

shifted green fluorescent protein and a blue-shifted green fluorescent protein. Claim 5 recites that the phosphorylation region comprises the amino acid sequence of SEQ ID NO:1. Claim 9 is directed to a method that employs the monitor protein.

The Office Action asserts that the claims are not adequately described because the specification does not disclose identifying characteristics of the phosphorylation region that are critical for a monitor protein to exhibit a change in fluorescence intensity as a result of phosphorylation of its phosphorylation region. (Page 5, lines 3-6.) The Office Action also asserts that the claims are not adequately described because the specification does not disclose identifying characteristics of the pair of fluorescent proteins that are critical for changes in fluorescence of the monitor protein as a result of phosphorylation of the phosphorylation region. (Page 5, lines 10-12.) The Office Action concludes that "the monitor protein of invention has to be identified by its amino acid structure as not all the members of the genus of proteins comprising part (a) and (b) as claimed in claim 13 are monitor proteins." (Page 8, lines 6-8.)

To satisfy the written description requirement, the specification must describe the claimed invention in sufficient detail that it clearly allows those of ordinary skill in the art to recognize that the invented the claimed subject matter. *In re Gosteli*, 872 F.2d. 1614, 1618 (Fed. Cir. 1989). Even if the Patent Office considers the subject matter of the claims to be broader than that disclosed in the originally filed specification, the written description requirement may be satisfied if the broader concept would naturally occur to one of skill in the art. *In re Smythe*, 480 F.2d 1376, 1384 (C.C.P.A. 1973). Possession of a genus can be demonstrated by describing a representative number of species of a genus by (1) actual reduction to practice, (2) reduction to drawings, or by (3) disclosure of relevant identifying characteristics. *The Regents of the*

University of California v. Eli Lilly and Company 119 F.3d 1559 (Fed. Cir. 1997). A representative number is an inverse function of the skill and knowledge in the art. See M.P.E.P. § 2163(II)(A)(3)(a)(ii). If the genus lacks variation, a single species may adequately describe the genus. See *In re Rasmussen*, 650 F.2d 1212 (CCPA 1981).

To advance prosecution, claim 13 has been amended to recite that the monitor protein comprises a phosphorylation region that "undergoes a conformational change upon phosphorylation" and that "the conformational change causes a change of the intensity of emitted fluorescence of the monitor protein." The amendment clarifies that the claims encompass only phosphorylation regions that undergo a conformational change upon phosphorylation, causing a change of the intensity of emitted fluorescence of the monitor protein. Similarly, the pending claims only encompass pairs of fluorescent proteins that change the intensity of emitted fluorescence of the monitor protein as a result of a conformational change in the phosphorylation region. Thus, the claims have been amended to only encompass operable monitor proteins and the scope of the claims is commensurate with the scope of the written description.

Furthermore, the genus of "a pair of fluorescent proteins" is not so variable as to require the description of more than one species in the specification. Pairs of fluorescent proteins capable of changing the intensity of emitted fluorescence of a monitor protein upon phosphorylation were known in the art before the effective filing date of the application, September 2, 1998. As applicants demonstrated in the response to Office Action dated September 8, 2003, at least six such pairs of fluorescent proteins, other than the pair disclosed in the specification, were known in the art. Tsien *et al.* teaches five pairs of these fluorescent proteins:

- S65C, a GFP containing a S65C amino acid substitution relative to wild-type GFP, and Y66H/Y145F, a BSGFP containing amino acid substitutions Y66H and Y145F relative to wild-type GFP;
- S65C, a GFP containing a S65C amino acid substitution relative to wild-type GFP, and P4, a BSGFP having amino acid substitution Y66H relative to wild-type GFP;
- S65T, a GFP having an S65T amino acid substitution relative to wild-type GFP, and W7, a BSGFP containing Y66W, N146L, M153T, V163A, and N212K relative to wild-type GFP;
- P4-3, a BSGFP containing amino acid substitutions Y66H and Y145F relative to wild-type GFP, and W7, a BSGFP containing Y66W, N146L, M153T, V163A, and N212K relative to wild-type GFP; and
- W1B, a BSGFP having F64L, S65T, Y66W, N146I, M153T, V163A, and N212K amino acid substitutions relative to wild-type GFP, and 10c, a yellow fluorescent protein having S65G, V68L, V72A, and T203Y amino acid substitutions relative to wild-type GFP.

U.S. Patent 5,981,200, column 24, lines 31-62. Day teaches a sixth pair of fluorescent proteins. Day teaches that a BFP, containing amino acid residue substitutions Y66H, Y145F relative to wild-type GFP and a GFP, having a S65T amino acid substitution relative to wild-type GFP are bound to opposite ends of a protein and are capable of changing the emitted fluorescence of the protein. (*Molecular Endocrinology* (1998) *12*:1410-1419). Page 1414, column 2, lines 10-13.

Thus, the specification describes one species of the "pair of fluorescent proteins." Six other such pairs of fluorescent proteins were known in the art at the time the application was filed. Based on the specification's disclosure of a pair of fluorescent proteins, the broader concept of pairs of fluorescent proteins would have naturally occurred to one of skill in the art. Thus, one of skill in the art would have understood that applicants had possession of the genus of pairs of fluorescent proteins in the claimed monitor protein.

The specification also discloses a representative number of species of the "phosphorylation region that undergoes a conformational change" of the claimed monitor

proteins. The specification discloses that the CREB phosphorylation sequence of SEQ ID NO:1 is a representative species of the genus of phosphorylation regions. The specification discloses that a "conformational change was generated by phosphorylation of the CREB phosphorylation sequence, allowing RSGFP and BSGFP at either end of the CREB sequence to interfere with each other, emitting fluorescence." (Page 24, lines 1-4.) In addition, phosphorylation regions "that undergo a conformational change upon phosphorylation" were known in the art before the effective filing date of the application, September 2, 1998. See response to Office Action filed September 8, 2003 and attached exhibits C-J. In summary:

- Chu teaches that phosphorylation of serine residues on the cGMP-dependent protein kinase (PKG) induces a conformational change in the protein. (Exhibit C.)
- Barth teaches that a structural change is induced in the sarcoplasmic reticulum Ca^{2+}-ATPase following phosphorylation. (Exhibit D.)
- Djordjevic teaches that phosphorylation of *S. typhimurium* CheB methylesterase induces a conformational change in the protein. (Exhibit E.)
- Ulitzur teaches that phosphorylation induces a conformational change in mapmodulin. (Exhibit F)
- Zhang teaches that the C-terminal domain of mouse RNA polymerase II (CTD) undergoes a conformational change upon phosphorylation. (Exhibit G.)
- Hubbard teaches that IRK and a specific peptide portion of IRK are phosphorylated. Hubbard teaches that phosphorylation of IRK or the peptide portion of IRK induces a conformational change. (Exhibit H.)
- Ma teaches that a polypeptide containing the R domain of cystic fibrosis transmembrane conductance regulator (CFTR) protein undergoes a conformational change following phosphorylation. (Exhibit I.)
- Drake teaches that phosphorylation of CheY protein at aspartic acid residue 57 induces a conformational change. (Exhibit J.)

Thus, many proteins and portions of proteins that undergo a conformational change as a result of phosphorylation were disclosed in the specification or were known in the art at the time of the effective filing date of the application. Based on the specification's disclosure, the broader concept of phosphorylation regions, as evidenced in Exhibits C-J, would have naturally occurred

to one of skill in the art. One of skill in the art would have understood that applicants had possession of the genus of phosphorylation regions in the claimed monitor proteins.

The specification's disclosure and the knowledge in the art prior to the effective filing date of the application demonstrate that one of skill in the art would have recognized that applicants had possession of the claimed monitor proteins comprising a pair of fluorescent proteins and a phosphorylation region.

Applicants respectfully request withdrawal of this rejection.

The Rejection of Claims 4, 5, 9, and 13 Under 35 U.S.C. § 112, First Paragraph

Claims 4, 5, 9, and 13 are rejected under 35 U.S.C. § 112, first paragraph as not being enabled for their full scope. The rejection is respectfully traversed.

To satisfy the enablement requirement, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation. *In re Wright*, 999 F.2d 1557, 1561 (Fed. Cir. 1993). That some experimentation may be required is not fatal; the issue is whether the amount of experimentation required is "undue." *In re Vaeck*, 947 F.2d 488, 495 (Fed. Cir. 1991). The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104 (Fed. Cir. 1985). The test is not merely quantitative, because a considerable amount of experimentation is permissible if the experimentation is merely routine or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988).

The Office Action asserts that the claims are not enabled because:

> The disclosure does not teach how to select [a] phosphorylation region and fluorescent proteins. Thus, one skilled in the art is forced to make a DNA construct encoding a phosphorylated region flanked by fluorescent proteins, express said construct and test the protein for the property of being phosphorylated as well as being able to change the fluorescence in response to phosphorylation. Without further guidance on the part of Applicants as to the nature and structure of the phosphorylation region and fluorescent proteins and the means of their connection, i.e. full structural description, the fusion protein that is the monitoring protein as well, experimentation left to those in the art is improperly extensive and undue.

Final Office Action, page 10, lines 2-11.

As discussed above, independent claim 13, from which claims 4, 5, and 9 depend, has been amended to recite that the monitor proteins comprise a "phosphorylation region that undergoes a conformational change upon phosphorylation" and a "pair of fluorescent proteins . . . wherein the conformational change causes a change of the intensity of emitted fluorescence of the monitor protein." Thus, claim 13, as amended, comprises monitor proteins that encompass only phosphorylation regions that undergo a conformational change as a result of phosphorylation and only pairs of fluorescent proteins that cause a change of the intensity of emitted fluorescence of the monitor protein as a result of the conformational change of the phosphorylation region. The specification and the knowledge available to one of skill in the art at the effective filing date of the application were such that one of skill in the art would have been able to make and use these monitor proteins without undue experimentation.

The nature and structure of pairs of fluorescent proteins are described in the specification and were also known to those of skill in the art prior to the effective filing date of the application. The specification discloses that a pair of fluorescent proteins, RSGFP and BSGFP, displays a

change in fluorescence as a result of phosphorylation of the CREB peptide: "This indicates that [a] conformation[al] change was generated by phosphorylation of the CREB phosphorylation sequence, and thereby RSGFP and BSGFP at the either end could interfere [with] each other, emitting fluorescence." (Page 18, lines 22-25.) As indicated above, Tsien (column 24, lines 31-62) and Day (page 1414, column 2, lines 10-13) cumulatively teach six pairs of fluorescent proteins that are bound to opposite ends of a protein and that are capable of changing the emitted fluorescence of the protein. Thus, the teachings in the specification, combined with the knowledge available in the art, demonstrate that one of skill in the art would have been able to choose a "pair of fluorescent proteins" to make and use in the claimed monitor protein without undue experimentation.

As evidence that the specification's teachings and knowledge of those of skill in the art would have been sufficient to enable one of skill in the art to make and use the monitor proteins comprising a pair of fluorescent proteins, applicants submit a declaration under Rule 132. The declaration describes the production and testing of two additional monitor proteins comprising a pair of fluorescent proteins different from the pair of fluorescent proteins described in the specification. The pairs of fluorescent proteins described in the declaration comprise a yellow fluorescent protein (YFP) and a cyan fluorescent protein (CFP) or a YFP and a modified CFP (CFP-$_{ASK}$). Each of the pairs of fluorescent proteins flanked the CREB protein phosphorylation region designated as ART (amino acid residues EILSRRPSYRKILNDLSSD). See paragraph 2. These were tested for monitor protein activity by determining whether they exhibited a change in intensity of emitted fluorescence as a result of phosphorylation using fluorescence resonance energy transfer (FRET).

The YFP-ART-CFP and YFP-ART-CFP-$_{ASK}$ proteins each exhibited a clear difference in fluorescence intensity emittance at FRET donor wavelength 477 and at FRET acceptor wavelength 528 that was dependent on phosphorylation by protein kinase A (PKA). See paragraph 7 and Figures 1 and 2.

The values for each proteins' FRET donor/acceptor ratio of emitted fluorescence intensity was also determined to further demonstrate that they function as monitor proteins. At FRET donor wavelength 477 nm, YFP-ART-CFP emitted fluorescence at an intensity of 68.87 in the absence of PKA and emitted fluorescence at a higher intensity of 74.86 in the presence of PKA. At FRET acceptor wavelength 528 nm YFP-ART-CFP emitted fluorescence at an intensity of 248.02 in the absence of PKA and emitted fluorescence at a lower intensity of 233.78 in the presence of PKA. The FRET donor/acceptor ratio (477 nm/528 nm) of fluorescence intensity based on these measured values changed from 0.2777 in the absence of PKA to 0.3202 in the presence of PKA. See paragraph 9 and Table 1.

At FRET donor wavelength 477 nm, YFP-ART-CFP-$_{ASK}$ emitted fluorescence at an intensity of 56.72 in the absence of PKA and emitted fluorescence at a higher intensity of 58.87 in the presence of PKA. At FRET acceptor wavelength 528 nm YFP-ART-CFP-$_{ASK}$ emitted fluorescence at an intensity of 211.60 in the absence of PKA and emitted fluorescence at a lower intensity of 191.45 in the presence of PKA. The donor/acceptor ratio based on these measurements changed from 0.2681 in the absence of PKA to 0.3075 in the presence of PKA. See paragraph 10 and Table 1.

The change of the ratios of intensity of emitted fluorescence exhibited by each of these pairs of fluorescent proteins in the purified proteins further demonstrates that one of skill in the

art can use different pairings of fluorescent proteins in a monitor protein without exercising undue experimentation.

One of skill in the art would also have been able to select a phosphorylation region that undergoes a conformational change upon phosphorylation for use in the claimed monitor protein. The nature and structure of these phosphorylation regions were also known to those of skill in the art prior to the effective filing date of the application. The specification discloses a CREB polypeptide that can be used as a phosphorylation region in the monitor protein. The specification discloses that a "fusion protein derived from pETIC-ART [a plasmid encoding a RSGFP-CREB phosphorylation sequence-BSGFP protein] showed the differences in absorption wavelength." (Page 5, lines 19-20.) Furthermore, before the effective filing date of the application, the art taught other proteins and polypeptides that could be used as "a phosphorylation region that undergoes a conformational change upon phosphorylation." As described above, Chu, Barth, Djordjevic, Ulitzur, Zhang, Hubbard, Ma, and Drake each teach a protein or a portion of a protein that undergoes a conformational change as a result of phosphorylation. Thus, the specification and the knowledge available to those of skill in the art at the time the application was filed were such that the skilled artisan would have been able to choose a "phosphorylation region that undergoes a conformational change upon phosphorylation" to use in the monitor protein of claim 13.

Furthermore, it would not require undue experimentation for one of skill in the art to produce and test the claimed monitor proteins. The Office Action asserts, "The undue experimentation comprises making a DNA construct encoding a phosphorylated region flanked by fluorescent proteins, expressing said construct and testing the fusion protein for the property

of being phosphorylated as well as being able to change the fluorescence in response to phosphorylation." (Final Office Action dated February 9, 2004, page 11, lines 3-7.) In fact, any experimentation required to produce and test putative monitor proteins is either routine for those of skill in the art or is taught by the specification. The Patent Office has acknowledged that methods of producing fusion proteins were well known and routinely practiced in the art at the time the application was filed: "synthesis of hybrid molecules [was] well known in the art." (Paper 9, page 6, lines 17-18.) In addition, the specification teaches how to test if an amino acid sequence can be used as a phosphorylation region. This test would be routine for one of skill in the art to perform. The specification teaches that this test is performed by inserting the phosphorylation sequence between RSGFP and BSGFP and performing phosphorylation measurements using both [γ-^{32}P]ATP and those based on fluorescence change." (Page 19, lines 10-13.) If the inserted phosphorylation sequence can be phosphorylated in the [γ-^{32}P]ATP assay but no change in the intensity of emitted fluorescence is observed in the fluorescence assay, it means that the phosphorylation sequence is phosphorylated but does not undergo a conformational change that causes a pair of fluorescent proteins to interact with each other so as to emit a change in fluorescence. The specification provides detailed instructions for performing the [γ-^{32}P]ATP assay in Example 4 entitled, "Measurement of phosphorylation using γ-^{32}P." (See page 17, lines 7-33.) The specification also provides detailed instruction for performing fluorescence measurements in Example 5 entitled, "Measurement of phosphorylation by fluorescence change." (See page 17, line 35 to page 19, line 13.) Using the guidance from these specification teachings, one of skill in the art could readily test if a phosphorylation sequence can be used as a phosphorylation region. Thus, even if, *arguendo*, a considerable amount of

experimentation would be required to make and test the monitor proteins of claim 13, the experimentation required to do so would be merely routine to those of skill in the art. Therefore, any experimentation required to make and use the claimed monitor proteins would not be undue. The claims are therefore enabled.

Evidence that the specification's teachings and knowledge of those of skill in the art would have been sufficient to enable one of skill in the art to make and use the monitor proteins comprising a phosphorylation region is provided in the declaration submitted under Rule 132. The declaration describes the production and testing of three monitor proteins comprising a phosphorylation region different from the phosphorylation described in the specification. The phosphorylation regions described in the declaration comprise the ART having two additional amino acid residues (ART-CR), four additional amino acid residues (ART-CR-F14), or six additional amino acid residues (ART-CR-F16). Each of these phosphorylation regions was flanked by the YFP and CFP pair of fluorescent proteins. See paragraph 2.

Like the YFP-ART-CFP and YFP-ART-CFP-$_{ASK}$ proteins, each of these monitor proteins exhibited a clear difference in fluorescence intensity emittance at FRET donor wavelength 477 and at FRET acceptor wavelength 528 that was dependent on the presence of phosphorylation by protein kinase A (PKA). See paragraph 7 and Figures 3-5.

The values for each proteins' FRET donor/acceptor ratio of emitted fluorescence intensity was also determined to further demonstrate that they function as monitor proteins. At FRET donor wavelength 477 nm, YFP-ART-CR-CFP emitted fluorescence at an intensity of 103.81 in the absence of PKA and emits fluorescence at a higher intensity of 103.17 in the presence of PKA. At FRET acceptor wavelength 528 nm YFP-ART-CR-CFP emitted

fluorescence at an intensity of 146.40 in the absence of PKA and emits fluorescence at a lower intensity of 137.15 in the presence of PKA. The donor/acceptor ratio for the YFP-ART-CR-CFP protein changed from 0.7091 in the absence of PKA to 0.7523 in the presence of PKA. See paragraph 11 and Table 1.

At FRET donor wavelength 477 nm, YFP-ART-CR-F14-CFP emitted fluorescence at an intensity of 78.58 in the absence of PKA and emitted fluorescence at a higher intensity of 80.45 in the presence of PKA. At FRET acceptor wavelength 528 nm YFP-ART-CR-F14-CFP emitted fluorescence at an intensity of 105.87 in the absence of PKA and emitted fluorescence at a lower intensity of 100.38 in the presence of PKA. The donor/acceptor ratio for the YC-ART-CR-F14 protein changed from 0.7422 in the absence of PKA to 0.8014 in the presence of PKA. See paragraph 12 and Table 1.

At FRET donor wavelength 477 nm, YFP-ART-CR-F16-CFP emitted fluorescence at an intensity of 88.91 in the absence of PKA and emitted fluorescence at a higher intensity of 90.63 in the presence of PKA. At FRET acceptor wavelength 528 nm YFP-ART-CR-F16-CFP emitted fluorescence at an intensity of 112.43 in the absence of PKA and emitted fluorescence at a lower intensity of 106.16 in the presence of PKA. The donor/acceptor ratio for the YC-ART-CR-F16 protein changed from 0.7908 in the absence of PKA to 0.8538 in the presence of PKA. See paragraph 13 and Table 1.

The change in the intensity of emitted fluorescence for each of these proteins demonstrates that they have monitor protein activity. Thus, the declaration demonstrates that one of skill in the art would be able to alter the phosphorylation region of a monitor protein to produce new monitor proteins without undue experimentation.

Applicants respectfully request withdrawal of this rejection.

Respectfully submitted,

Date: 08-09-04

By: Sarah A Kagan

Sarah A. Kagan
Registration No. 32,141

BANNER & WITCOFF
1001 G Street, N.W., Eleventh Floor
Washington, D.C. 20001-4597
(202) 824-3000

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:	)	
	)	Group Art Unit: 1652
M. Hagiwara *et al.*	)	
	)	Examiner: M. Walicka
Serial No. 09/786,317	)	
	)	
Filed: April 27, 2001	)	Atty. Dkt. No. 004276.00002

For: **MONITOR PROTEIN FOR MEASURING PHOSPHORYLATION OF PROTEIN**

REQUEST FOR ADMITTANCE OF DECLARATION UNDER 37 C.F.R. § 1.195

U.S. Patent and Trademark Office
220 20th Street S.
Customer Window, Mail Stop AF
Crystal Plaza Two, Lobby, Room 1B03
Arlington, VA 22202

Sir:

This paper is filed in response to the final Office Action mailed February 9, 2004. A Notice of Appeal is also being filed. Thus, we believe no fees are due to make this response timely filed. If any fee is due, please charge our Deposit Account No. 19-0733.

Please admit the declaration under 37 C.F.R. §1.132.

Remarks

The declaration was not submitted earlier because applicants believed that the amendment and exhibits filed in response to the Office Action dated March 11, 2003 were sufficient to overcome an enablement rejection of the claims. In the final Office Action the

Patent Office more clearly stated its position as to why the claims are not enabled. The final Office Action states:

> The undue experimentation comprises making a DNA construct encoding a phosphorylated region flanked by fluorescent proteins, expressing said construct and testing the fusion protein for the property of being phosphorylated as well as being able to change the fluorescence in response to phosphorylation.

Final Office Action at page 11, lines 3-7. The Declaration under Rule 132 provides evidence that the monitor proteins of the claimed invention are enabled. The Declaration demonstrates that changing either (1) the phosphorylation region of a monitor protein to a different phosphorylation region or (2) one of the pair of fluorescent proteins of a monitor protein to a different fluorescent protein does not change the operability of the monitor protein, *i.e.*, to exhibit a phosphorylation-dependent change in emitted fluorescence intensity as a function of wavelength. Thus, the Declaration provides evidence that undue experimentation would not be required for one of skill in the art to make and use the claimed monitor proteins.

Entry of the declaration under Rule 132 is respectfully requested.

Respectfully submitted,

Date: August 9, 2004

By: _____

Sarah Kagan
Registration No. 32, 141

BANNER & WITCOFF
1001 G Street, N.W., Eleventh Floor
Washington, D.C. 20001-4597
(202) 824-3220

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	Group Art Unit: 1652
M. Hagiwara, *et al.*	)	
	)	Examiner: M. Walicka
Serial No. 09/786,317	)	
	)	Atty. Dkt. No. 04276.00002
Filed: March 2, 2001	)	

For: **MONITOR PROTEIN FOR MEASURING PROTEIN PHOSPHORYLATION**

DECLARATION UNDER 37 C.F.R. § 132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Masatoshi Hagiwara, declare as follows:

1. I am an inventor of the subject matter disclosed and claimed in the patent application identified above.

2. Under my direction, experiments were conducted that successfully produced additional monitor proteins comprising a pair of fluorescent proteins and a phosphorylation region. The following monitor proteins were produced and tested:

First Fluorescent Protein	Amino Acid Sequence of Phosphorylation Region, Derived from Creb Protein	Second Fluorescent Protein	Abbreviated Monitor Protein Name
YFP[1]	EILSRRPSYRKILNDLSSD	CFP[2]	YC-ART
YFP	EILSRRPSYRKILNDLSSD	CFP-$_{ASK}$[3]	YCA-ART
YFP	NSESVDSVTDSQKRREILSRRPSYRKILN DLSSDARSMV	CFP	YC-ART-CR
YFP	NSEDSQESVDSVTDSQKRREILSRRPSYR KILNDLSSDARSMV	CFP	YC-ART-CR-F14
YFP	NSESEDSQESVDSVTDSQKRREILSRRPS YRKILNDLSSDARSMV	CFP	YC-ART-CR-F16

[1] YFP=Yellow Fluorescent Protein containing amino acid substitutions V68L and Q69K.
[2] CFP=Cyan Fluorescent Protein
[3] CFP-$_{ASK}$=CFP containing an N-terminal Ala residue in place of naturally occurring Met-Val

3. A vector encoding each monitor protein was produced as follows. A cDNA encoding the YFP used in the monitor protein was obtained by subcloning a YFP gene from a commercially available vector (Clontech, Palo Alto, CA) and subsequently performing site mutagenesis on the cDNA such that it encoded V68L-Q69K YFP. A cDNA encoding CFP was also obtained from a commercially available vector (Clontech, Palo Alto, CA). To obtain a cDNA encoding CFP-$_{ASK}$, the nucleotide sequence encoding CFP was altered to encode an N-terminal Ala-Ser-Lys amino acid sequence in place of the naturally-occurring Met-Val-Ser-Lys. The YFP and CFP, or YFP and CFP-$_{ASK}$ nucleotide sequences were cloned into *E. coli* expression vector pET28 (Novagen, Madison, WI) flanking a nucleotide sequence encoding a phosphorylation region derived from CREB protein, EILSRRPSYRKILNDLSSD (ART).

4. The pET28 vector encoding YC-ART was further modified to increase the number of amino acid residues in the ART phosphorylation region between YFP and CFP. The nucleic acids encoding the additional amino acid residues in the ART phosphorylation region, like the nucleic acids encoding the ART phosphorylation region itself, were derived from CREB protein. YC-ART-CR has two additional amino acids, YC-ART-CR-F14 has six additional amino acid residues, and YC-ART-CR-F16 has eight additional amino acid residues relative to the ART phosphorylation region encoded by YC-ART.

5. The monitor proteins were expressed and purified as follows. Each of the pET28 expression vectors encoding a monitor protein was separately transformed into *E. coli* strain BL21. Expression of the monitor proteins was induced by addition of 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) to *E. coli* cultures. After culturing for 20 hr at 23 degrees Celsius, the cells were harvested and the monitor proteins were purified using Ni-Agarose resin. The pET28 expression vector provides a histidine tag fused at the carboxyl terminus of an expressed

protein to facilitate purification using Ni-Agarose resin.

6. We determined the ability of each of the expressed and purified monitor proteins to change the intensity of emitted fluorescence as a result of phosphorylation. Each of the purified monitor proteins (20 μg each) was incubated with 20 mM Tris-HCl pH 7.9, 1 mM ATP, 10 mM $MgCl_2$, and 0 or 400 ng PKA in total volume of 200 μl at room temperature for 30 minutes. Fluorescence resonance energy transfer (FRET) was measured under the excitation of 430-440 nm (emission 450-550 nm) using the FP-750 spectrofluorometer (Jasco, Tokyo, Japan).

7. We measured the fluorescence intensity emittance of the expressed and purified monitor proteins as a function of phosphorylation. The effect of adding 0.4 μg protein kinase A (PKA) to each of the expressed and purified monitor proteins on fluorescence intensity emittance as a function of wavelength was plotted. Each monitor protein exhibits a clear difference in fluorescence intensity emittance at fluorescence resonance energy transfer (FRET) donor wavelength 477 nm and at FRET acceptor wavelength 528 nm. Figure 1, below, is the plot obtained for protein YC-ART. Figure 2 is the plot obtained for protein YCA-ART. Figure 3 shows the plot obtained for protein YC-ART-CR. Figure 4 is the plot for protein YC-ART-CR-F14. Figure 5 is the plot for protein YC-ART-CR-F16.

Figure 1 (YC-ART)

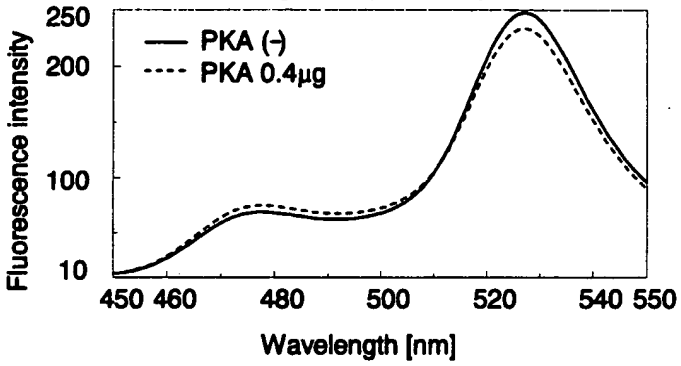


Figure 2 (YCA-ART)

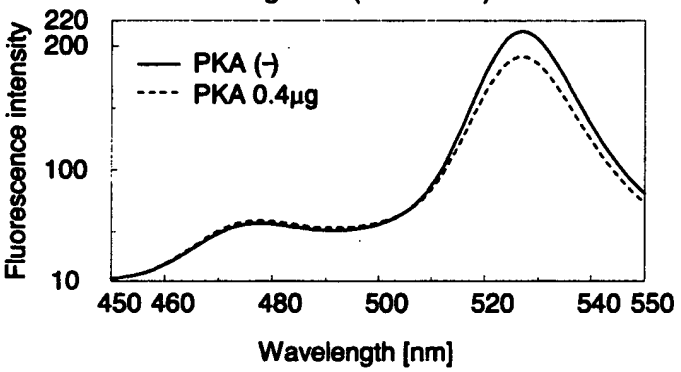


Figure 3 (YC-ART-CR)

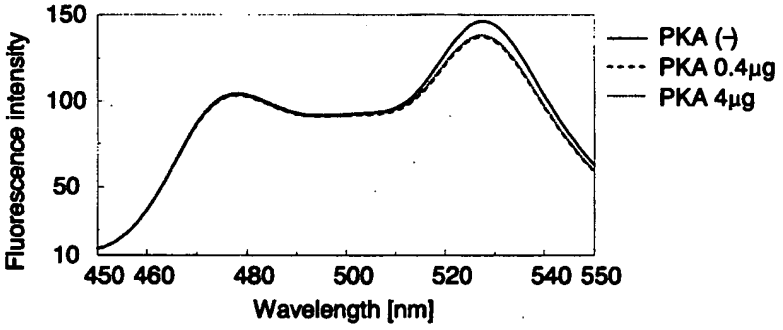


Figure 4 (YC-ART-CR-F14)

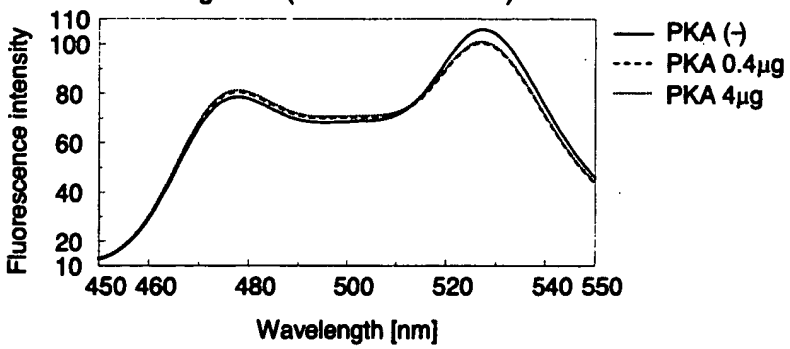
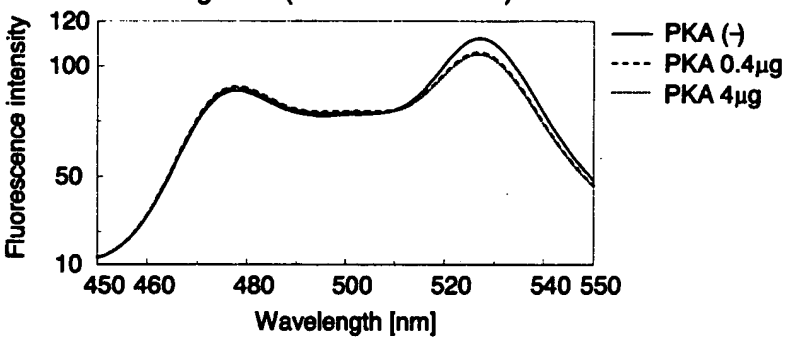


Figure 5 (YC-ART-CR-F16)



8. As shown in Figures 1-5, phosphorylation of each of the purified proteins causes a change in emitted fluorescence intensity. Thus, each of the purified proteins can function as a monitor protein for testing protein phosphorylation reactions.

9. The difference in fluorescence intensity emittance ratio for each purified protein in the presence versus the absence of 0.4 μg PKA further demonstrates that each purified protein functions as a monitor protein. At FRET donor wavelength 477 nm, YC-ART emits fluorescence at an intensity of 68.87 in the absence of PKA and emits fluorescence at a higher intensity of 74.86 in the presence of PKA. At FRET acceptor wavelength 528 nm YC-ART emits fluorescence at an intensity of 248.02 in the absence of PKA and emits fluorescence at a lower intensity of 233.78 in the presence of PKA. The FRET donor/acceptor ratio (477 nm/528

nm) of fluorescence intensity based on these measured values changes from 0.2777 in the absence of PKA to 0.3202 in the presence of PKA. See Table 1.

Table 1

Monitor Protein	PKA added to reaction	Intensity of fluorescence emitted at 477nm	Intensity of fluorescence emitted at 528nm	Donor/acceptor ratio of fluorescence intensity 477/528
YC-ART	0 μg	68.8705	248.0250	0.2777
YC-ART	0.4 μg	74.8682	233.7850	0.3202
YCA-ART	0 μg	56.7294	211.6000	0.2681
YC-ART	0.4 μg	58.8798	191.4560	0.3075
YC-ART-CR	0 μg	103.8170	146.4010	0.7091
YC-ART-CR	0.4 μg	103.1770	137.1540	0.7523
YC-ART-CR-F14	0 μg	78.5804	105.8700	0.7422
YC-ART-CR-F14	0.4 μg	80.4551	100.3880	0.8014
YC-ART-F16	0 μg	88.9173	112.4340	0.7908
YC-ART-F16	0.4 μg	90.6399	106.1600	0.8538

10. At FRET donor wavelength 477 nm, YCA-ART emits fluorescence at an intensity of 56.72 in the absence of PKA and emits fluorescence at a higher intensity of 58.87 in the presence of PKA. At FRET acceptor wavelength 528 nm YCA-ART emits fluorescence at an intensity of 211.60 in the absence of PKA and emits fluorescence at a lower intensity of 191.45 in the presence of PKA. The donor/acceptor ratio based on these measurements changes from .2681 in the absence of PKA to .3075 in the presence of PKA. See Table 1.

11. At FRET donor wavelength 477 nm, YC-ART-CR emits fluorescence at an intensity of 103.81 in the absence of PKA and emits fluorescence at a higher intensity of 103.17 in the presence of PKA. At FRET acceptor wavelength 528 nm YC-ART-CR emits fluorescence at an intensity of 146.40 in the absence of PKA and emits fluorescence at a lower intensity of 137.15 in the presence of PKA. The donor/acceptor ratio for the YCA-ART-CR protein changes from .7091 in the absence of PKA to .7523 in the presence of PKA. See Table 1.

12. At FRET donor wavelength 477 nm, YC-ART-CR-F14 emits fluorescence at an intensity of 78.58 in the absence of PKA and emits fluorescence at a higher intensity of 80.45 in the presence of PKA. At FRET acceptor wavelength 528 nm YC-ART-CR-F14 emits fluorescence at an intensity of 105.87 in the absence of PKA and emits fluorescence at a lower intensity of 100.38 in the presence of PKA. The donor/acceptor ratio for the YC-ART-CR-F14 protein changes from .7422 in the absence of PKA to .8014 in the presence of PKA. See Table 1.

13. At FRET donor wavelength 477 nm, YC-ART-CR-F16 emits fluorescence at an intensity of 88.91 in the absence of PKA and emits fluorescence at a higher intensity of 90.63 in the presence of PKA. At FRET acceptor wavelength 528 nm YC-ART-CR-F16 emits fluorescence at an intensity of 112.43 in the absence of PKA and emits fluorescence at a lower intensity of 106.16 in the presence of PKA. The donor/acceptor ratio for the YC-ART-CR-F16 protein changes from .7908 in the absence of PKA to .8538 in the presence of PKA. See Table 1.

14. Each of the purified proteins (YC-ART, YCA-ART, YC-ART-CR, YC-ART-CR-F14, and YC-ART-CR-F16) exhibits a measurable phosphorylation-dependent change in intensity of emitted fluorescence as a function of wavelength. Thus, each of these proteins functions as a monitor protein.

15. Changing one of the pair of fluorescent proteins used in the purified proteins did not alter their ability to function as monitor proteins. YC-ART and YCA-ART differ in one of the pair of fluorescent proteins flanking the ART phosphorylation region. As explained above, both proteins exhibited a phosphorylation-dependent change in fluorescence intensity as a function of wavelength. See Figures 1 and 2, and Table 1. Thus, one of skill in the art can use different pairings of fluorescent proteins in a monitor protein.

16. Changing the phosphorylation region used in the purified proteins also did not

alter the ability to function as monitor proteins. YC-ART, YC-ART-CR, YC-ART-CR-F14, and YC-ART-CR-F16 differ in the number of amino acid residues present in the phosphorylation region flanked by YFP and CFP fluorescent proteins. Despite this difference, each of these purified proteins functions as a monitor protein because each exhibits a phosphorylation-dependent change in emitted fluorescence intensity as a function of wavelength. See Figure 1, 3, 4, and 5, and Table 1. Thus, one of skill in the art can alter the phosphorylation region of a monitor protein to produce new monitor proteins.

17. One of skill in the art can readily and predictably produce monitor proteins, as shown in paragraphs 1-16, that comprise a pair of fluorescent proteins and a phosphorylation.

18. I declare that all statements made herein of my own knowledge are true and that I believe all statements made on information and belief are true and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

8/5/2004

Date

Masatoshi Hagiwara